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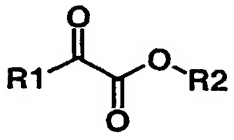
(54) Title: PYRUVATE ESTER COMPOSITION AND METHOD OF USE FOR RESUSCITATION AFTER EVENTS OF ISCHEMIA AND REPERFUSION



R_1 = Methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tertbutyl, pentyl, 4-methylpentyl, 3-methylpentyl, hexyl, heptyl, octyl, 1-phenylmethyl, 2-phenyl-ethyl;

R_2 = Ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tertbutyl, pentyl, 4-methylpentyl, ethoxymethyl, 2-ethoxyethyl, carboxymethyl, ethoxycarbonylmethyl.

(57) Abstract: A therapeutic composition comprising an alkyl, aralkyl, alkoxyalkyl, or carboxyalkyl ester of 2-ketoalkanoic acid and a component for inducing and stabilizing the enol resonance form of the ester at physiological pH values is disclosed. The composition of the invention further comprises a pharmaceutically acceptable carrier vehicle in which the enol resonance form of the ester is stabilized at physiological pH values. Formulations containing the compositions of the invention permit the successful use of 2-ketoalkanoic acid esters, e.g., pyruvic acid esters, to treat, e.g., ischemic events, shock, organ reanimation, resuscitation and other recognized pyruvate-effective treatments. The compositions of the inventions are also useful in a process for preserving organ parts, organs or limbs removed from a living mammal and in need of preservation, e.g., for later transplantation to an organ recipient. The figure shows the structures of the preferred 2-ketoalkanoic acid esters in the composition of the invention.

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TITLE OF THE INVENTION

PYRUVATE ESTER COMPOSITION AND METHOD OF USE FOR RESUSCITATION AFTER EVENTS OF ISCHEMIA AND REPERFUSION

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the priority of U.S. Provisional Application No. 60/158,091 filed October 7, 1999 entitled, PYRUVATE ESTER COMPOSITION AND METHOD OF USE FOR RESUSCITATION AFTER ISCHEMIA AND REPERFUSION, the whole of which is hereby incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Part of the work leading to this invention was carried out with United States Government support provided under a grant from the National Institutes of Health, Grant No. GM37631. Therefore, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

This invention relates to several new pyruvate compounds and methods for resuscitation and reanimation of mammals, especially humans, before, during and after, e.g., (1) mesenteric ischemia, mesenteric thrombus or mesenteric venous occlusion; (2) aortic aneurism repair, coronary artery bypass, surgical treatment of arterial occlusion of limbs; (3) hemorrhagic shock, resulting from

either penetrating and blunt trauma; and (4) preservation and transplantation of organs. Ischemia is defined herein as the interruption of oxygen supply, via the blood, to an organ or to part of an organ. Examples of ischemic events include (i) myocardial, cerebral, or intestinal infarction following obstruction of a branch of a coronary, cerebral, or mesenteric artery, and (ii) removal and storage of an organ prior to transplantation. In the case of myocardial infarction, prompt restoration of blood flow to the ischemic myocardium, i.e. coronary reperfusion, is a key component of the treatment. This is because mortality is directly related to infarct size (tissue necrosed) which is related to the severity and duration of the ischemic event. The consequences of hemorrhagic shock are similar to those of ischemia, although the causative event is not an interruption of blood flow but rather the event of massive blood loss itself which causes deprivation of the oxygen supply.

Notwithstanding the need to supply an organ cut-off from a normal blood supply with oxygen, it has been found that reperfusion injury may occur upon restoration of blood flow. This results from-the production of reactive oxygen species (ROS), namely, hydrogen peroxide, hydroxyl radicals and superoxide radicals, among others, which are formed from both extracellular and intracellular sources. ROS are highly reactive species that, under normal conditions, are scavenged by endogenous defense mechanisms. However, under conditions of post-ischemic oxidative stress, ROS interact with a variety of cellular components, causing peroxidation of lipids, denaturation of proteins, and interstitial matrix damage and resulting

in increase of membrane permeability and release of tissue enzymes.

In an attempt to minimize these undesirable side effects of perfusion in the treatment of ischemia and also of shock, researchers have demonstrated the utility of various antioxidants in the reperfusion process.

Banda et al. (1996), together with Kurose et al. (1997), suggested the use of an inhibitor of ROS production to protect the reperfused myocardium and the use of agents and inhibitors that reduce ROS levels. In a similar context, desiring to provide more efficient resuscitation, researchers have demonstrated the additive utility of incorporating an antioxidant and a beneficial metabolic fuel into the reperfusion regimen. Salahudeen et al. (1991) used solutions of pyruvate, an ROS scavenger and a metabolically important precursor fuel for gluconeogenesis, to protect against hydrogen peroxide induced acute renal failure. Cicalese et al. (1996) found that pretreatment with intraluminal pyruvate ameliorates post ischemic small bowel injury while Crestanello et al. (1998), DeBoer et al. (1993), and O'Donnell-Tormey et al. (1987) have substantiated this finding by examining the ameliorative effects of both endogenously secreted pyruvate and exogenously added material in the reperfusion and subsequent function of organ and tissue preparations subjected to ischemia and simulated shock. Varma et al. (1998), similarly, have shown that in a cultured lens system, after exposure of the cultured lens to free radical oxidant stress, pyruvate and its esters have certain cytoprotecting and restorative effects.

In a further effort directed to protecting reperfused heart tissue, U.S. Pat. No. 5,075,210, herein incorporated by reference, discloses a process for reperfusing a heart for transplantation. The patent discloses a cardioplegic solution containing sodium chloride, potassium chloride, calcium chloride, sodium bicarbonate, sodium EDTA, magnesium chloride, sodium pyruvate and a protein.

U.S. Pat. No. 5,294,641, herein incorporated by reference, is directed to the use of pyruvate to prevent the adverse effects of ischemia. The pyruvate is administered prior to a surgical procedure to increase a patient's cardiac output and heart stroke volume. The pyruvate is administered as a calcium or sodium salt. The pyruvate can alternatively be an amide of pyruvic acid such as ethylamino pyruvate. Similarly, U.S. Pat. No. 5,508,308, herein incorporated by reference, discloses the use of pyruvyl glycine to treat reperfusion injury following myocardial infarction.

U.S. Pat. No. 4,988,515 and 5,705,210, herein incorporated by reference, use pyruvate salts in cardioplegic solutions and in preservation solutions for the heart before transplantation. U.S. Pat. No. 4,970,143, herein incorporated by reference, discloses the use of acetoacetate for preserving living tissue, including addition of the pyruvate to the preservation solution.

U.S. Pat. No. 5,100,677 herein incorporated by reference, discloses the composition of various parenteral solutions. Of interest is a recommendation to include pyruvate anions (apparently from metal salts) in intravenous solutions.

U.S. Pat. No. 5,798,388, herein incorporated by reference, further describes the utility of pyruvate salts and of various complex derivatives, such as amides, for the treatment of ROS in the context of airway inflammation. The patent discloses a pyruvate compound in the form of a covalently linked pyruvoyl-amino acid. By utilizing this type of a pyruvate delivery system, the negative effect of pyruvate salt is avoided. However, administration of large amounts of pyruvate-amino acid may result in nitrogen overload which could harm patients with liver and/or kidney pathology.

In a similar context and based on a similar rationale for pyruvate delivery, U.S. Pat. No. 5,876,916 pertains to the utility of pyruvate thiolesters and polyol esters for the treatment or prevention of reperfusion injury following ischemia, diabetic effects, cholesterol levels, injured organs, ethanol intoxication or as a foodstuff; and U.S. Pat. No. 5,633,285; 5,648,380; 5,652,274; and 5,658,957, each herein incorporated by reference, disclose various compositions, salts, prodrugs and derivatives of pyruvate in mixtures with other antioxidants, fatty acids as anti-inflammatory and immunostimulating wound healing compositions. However, administration of large amounts of complex pyruvate-amino acid and other pro-drug derivatives requiring enzymatic hydrolysis prior to liberation of their antioxidant effects may result in nitrogen and/or other xenobiotic overload, which could harm patients directly, interfere with normal detoxifying processes, or cause toxic effects through by-products of limited shelf-life.

Notwithstanding the acceptance of pyruvate as an effective component of a reperfusion solution or other varied applications, pyruvic acid is a strong and unstable acid which cannot be infused as such. On standing in solution, pyruvic acid and its salts at various pH values, including in the physiological range, are known to form both a stable hydrate and a dimer (para-pyruvate), neither of which react with ROS as antioxidants and both of which are known inhibitors of pyruvate utilization as a metabolic fuel, thereby abrogating any of the beneficial effects which might have accrued from pyruvate administration in accordance with the prior art just described.

Furthermore, it has been recognized that traditional pharmacological pyruvate compounds, such as salts of pyruvic acid, are not particularly physiologically suitable. For example, these compounds lead to the accumulation of large concentrations of ions (e.g., calcium or sodium) in the patient's body fluids. Similarly, amino acid compounds containing pyruvate can lead to excessive nitrogen loads. It has also been proposed to infuse pyruvylglycine, the amide function of which is presumably hydrolyzed in plasma and/or tissues, thus liberating pyruvate.

However, at the high rates of pyruvoylglycine infusion required to achieve 1 mM pyruvate in plasma, the glycine load may be harmful to patients suffering from hepatic or renal pathologies. Also, flooding plasma with glycine may interfere with the transport of some amino acids across the blood-brain barrier. Accordingly, while potentially suitable to organ preservation, these pyruvate

compounds are less suited to treating an organ *in vivo*, and it is recognized that a need exists to provide a pyruvate delivery compound that is more physiologically acceptable.

There is also a recognized need to provide a pyruvate delivery system that is cost effective, simple, and devoid of opportunities for contamination because of 1) limited shelf-life, 2) complexity of formulation, 3) reactivity and co-reactivity with excipients and other formulation materials, 4) adverse biochemical reactivity during transport, translocation, and uptake into tissues, and 5) the requirement for metabolic activation via enzymatic hydrolysis by amidases or peptidases. Therefore, it would be desirable to have available an alternate physiologically compatible therapeutic pyruvate compound.

SUMMARY OF THE INVENTION

The invention described herein provides a new and improved, accessible composition for the above-indicated uses.

In one aspect, the invention is directed to a composition comprising an alkyl, aralkyl, alkoxyalkyl or carboxyalkyl ester of 2-ketoalkanoic acid and a component for inducing and stabilizing the enol resonance form of the ester at physiological pH values. The composition of the invention further comprises a pharmceutically acceptable carier vehicle in which the enol resonance form of the ester is stabilized at physiological pH values.

Preferably, the ester in the composition of the invention is an alkyl ester of 2-ketopropionic acid

(pyruvic acid), most preferably the ethyl ester, and the stabilizing component is a cationic material, preferably a divalent cation, and most preferably calcium or magnesium. The pharmaceutically acceptable carrier in the composition of the invention can be any carrier vehicle generally recognized as safe for administering a therapeutic agent to a mammal, e.g., a buffer solution for infusion, a tablet for oral administration or in gel, micelle or liposome form for on-site delivery. A preferred buffer solution is isotonic or hypertonic saline; or a bicarbonate, phosphate, plasma extender, microcolloid or microcrystalline solution. Particularly preferred is a Ringer's solution of isotonic saline supplemented with potassium ion. In a particularly preferred aspect, the composition of the invention comprises ethyl pyruvate admixed with calcium ion in a Ringer's solution at a pH in the range of 7-8.

In other aspects, the ester portion of the 2-ketoalkanoic acid ester compound in the composition of the invention is selected preferably from the group consisting of ethyl, propyl, butyl, carboxymethyl, acetoxymethyl, carbethoxymethyl and ethoxymethyl esters. The 2-ketoalkanoic acid portion is selected preferably from the group consisting of 2-keto-butyrate, 2-ketopentanoate, 2-keto-3-methyl-butyrate, 2-keto-4-methyl-pentanoate and 2-keto-hexanoate.

In another aspect, the invention is directed to methods for treating injuries, conditions or disorders associated with events such as ischemic events or reperfusion. Formulations containing the novel compositions of the invention permit the successful use of

2-ketoalkanoic acid esters, e.g., pyruvic acid esters, to treat, e.g., ischemic events, shock, organ reanimation, resuscitation and other recognized pyruvate-effective treatments as sufficiently high loads of pyruvate can be administered without a toxic constituent. Moreover, use of the compositions of the invention provides a direct replacement for traditional lactated Ringer's solutions uncomplicated by the addition of co-active ingredients or complex excipients, such as those comprised of multiple compounds or molecular derivatives of pyruvate itself. The compositions of the inventions are also useful in a process for preserving organ parts, organs or limbs removed from a living mammal and in need of preservation, e.g., for later transplantation to an organ recipient. Such processes are well known to those of skill in the art, e.g., as described in U.S. Patent No. 5,066,578, hereby incorporated by reference herein.

A further practical advantage of the methods of the invention is the formulation of the active 2-ketoalkanoic acid ingredient as a biologically safe, readily hydrolyzable ester which can be taken up into tissues and cells by diffussive processes through membranes, owing to said ester's greater lipophilicity over the corresponding salt, while retaining the ability to be hydrolyzed intracellularly by means of non-specific esterases and/or non-specific, marginally alkaline solvolysis catalyzed by organic acids or bases such as amino acid residues at physiological pH values.

More importantly, the method of this invention provides 2-ketoalkanoic acids, e.g., pyruvic acid, in a stabilized ester form that inactivates reactive oxygen

species by more than one mechanism of reaction and whose reaction products with reactive, hypervalent oxygen, such as hydrogen peroxide, affords degradation products that themselves are metabolic fuels instead of potentially harmful excretory products or metabolites.

BRIEF DESCRIPTION OF THE DRAWINGS

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims, taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows the structures of the preferred 2-ketoalkanoic acid esters in the composition of the invention;

Fig. 2 shows the structures of certain preferred esters in the composition of the invention, their enol resonance structures and the structures of certain prior art compounds;

Fig. 3 shows the system and computational parameters used for the measurement of mucosal-to-serosal intestinal permeability following practice of the method of the invention;

Fig. 4 shows the intestinal permeability results achieved for a control composition relative to compositions of the invention; and

Fig. 5 shows the results obtained for mucosal injury scores for compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

Accordingly, it is a primary object of this invention to provide new and improved compositions containing 2-

ketoalkanoic acid esters and methods of using them to treat certain conditions as described above.

To achieve the foregoing objects and in accordance with the purpose of the invention, as embodied and broadly described herein, one novel composition of this invention comprises a 2-ketoalkanoic acid ester, in accordance with the molecular structures shown in Fig. 1, admixed with a sufficient concentration of biologically safe organic or inorganic cations to induce enolization of the 2-keto functionality of the ester at physiological pH values. In a preferred embodiment, the composition comprises an alkyl ester of 2-ketopropionic acid (pyruvic acid), the ester is the ethyl analog and the cation is a divalent cation, particularly either calcium or magnesium. In a particularly preferred formulation of the composition of the invention, the ester compound is ethyl pyruvate admixed with calcium ion in a Ringer's solution at a pH of about 7-8.

The therapeutic compositions of the invention may be administered orally, topically, or parenterally, (e.g., intranasally, subcutaneously, intramuscularly, intravenously, intraluminally, intra-arterially, intravaginally, transurethrally or rectally) by routine methods in pharmaceutically acceptable inert carrier substances. For example, the therapeutic compositions of the invention may be administered in a sustained release formulation using a biodegradable biocompatible polymer, or by on-site delivery using micelles, gels, liposomes, or a buffer solution. The active ester agent in the composition of the invention can be administered, as an infusate, at a concentration of, e.g., 20-200 mM, at a rate of, preferably,

10-100 mg/kg/hr, in a buffer solution as described herein. In bolus form, the active ester agent can be administered at a dosage of, e.g., 10-200 mg/kg from 1-4 times daily. The cation in the composition of the invention is at an appropriate concentration to induce enolization of the 2-keto functionality of the amount of active ester agent in the administered composition. Optimal dosage and modes of administration can readily be determined by conventional protocols.

It is believed that pyruvate, and other 2-ketoalkanoic acids, when liberated intracellularly from the esters delivered, e.g., by the reanimation perfusate, acts as a NADH trap and a trap for ROS generated upon reperfusion. In the first instance, a 2-ketoalkanoic acid reacts to afford lactate, oxidizing excess NADH and thereby protecting against the "reductant stress" generated during the physiological insult caused by hypoxia. In the latter instance, a 2-ketoalkanoic acid reacts with hypervalent oxygen, as demostrated in the prior art, to form a transient peracid which decomposes spontaneously, and eventually, to acetate and carbon dioxide. The resulting acetate is a waste product, which may be salvaged by re-entry into the acetylCoA pool and harvested biochemically via intermediary metabolism in the Krebs cycle or via gluconeogenesis.

However, and more significantly for the purposes of this invention, the 2-ketoalkanoic acid ester itself serves as an antioxidant by a different mechanism, namely, via reaction with hypervalent oxygen at the enol methylene group. ROS is a membrane associated process, since hypervalent oxygen is generated by a redox cascade

mediated by cytochromes in the microsomes or the mitochodria. It is also an intracellular process that takes place in a lipophilic environment rather than in cytosol, and the thermodynamic properties of a 2-ketoalkanoic acid ester are such that its reactivity towards redox reaction in a lipophilic phase is putatively favored by the cation mediated keto-enol equilibrium. *Ab initio* and semi-empirical thermodynamic analyses on ethyl pyruvate as a representative enolizable molecule in the presence of calcium are discussed in greater detail as part of Example I below.

For example, using pyruvate as the exemplary 2-ketoalkanoic acid, formation of transient epoxides and subsequent rearrangement affords the corresponding hydroxylated pyruvate esters at the 3-carbon, by a mechanism similar to that of 3-hydroxy-pyruvate formation in intermediary metabolism as well as that of carbon additions to the phosphoenolpyruvate congener. Hydroxylation alpha to keto groups is also a recognized cytochrome mediated process in steroid metabolism and in microsomal hydroxylation of drugs. The resulting hydroxy-pyruvates, in turn, when solvolyzed into the carboxylic anions, can then react once again with hypervalent oxygen to afford hydroxyacetic acid (glycolic acid), the net result being that pyruvate esters can ultimately quench two equivalents of ROS while pyruvates are limited thermodynamically to quenching only one. As mentioned above, 2-ketoalkanoic acid esters other than pyruvate esters are also appropriate for use in compositions of the invention as long as the active compound is metabolizable as described above for the pyruvate ester.

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These examples are not intended in any way otherwise to limit the scope of the disclosure.

EXAMPLE 1

Thermodynamic modeling of pyruvate esters

Semiempirical quantum chemistry permits the comparative evaluation of various pyruvate analogs with regard to the properties that determine each molecule's reactivity. As one can note a marked difference in the biological effect of ethyl pyruvate versus sodium pyruvate as antioxidants, the hypothesis that these two molecules are thermodynamically different can be tested by Huckel Molecular Orbital (HMO) analysis followed by Complete Neglect of Differential Overlap Analysis (CNDO), using Molecular Modeling Pro/MOPAC software (ChemSW, Inc. Fairfield, CA). The following results were obtained for the structures shown in Fig. 2, after their conformations were set by energy minimization to the optimal conformation:

TABLE 1

Comparison of Thermodynamic Properties

Compound	Energy	Dipole	LogP	H-Acceptor	H-Donor
Na Pyruvate (1)	-31.7	355.6	-85.9	17.8	2.9
Na Pyruvate hydrate (2)	-16.5	462.8	-71.2	23.9	4.8
Na Enol-pyruvate (3)	-30.7	358.0	-72.1	17.7	2.8
Ethyl pyruvate (4)	-86.5	2.8	-.21	.73	8.5
Ethyl enol-pyruvate (5)	-84.1	2.5	-.37	.71	7.3
Ca enol ethyl pyruvate (6)	-82.3	2.7	-.41	.85	7.2

From the trend in minimization energies, the lower and, therefore, the more stable configurations are those associated with the pyruvate esters, although the differences all fall within an order magnitude. On the other hand, the esters show markedly lower dipole moments, reflecting their relatively weak ionization and dissociation potentials, a fact that is further supported by the higher LogP values, which are a measure of relative lipophilicity. Also, the esters are poorer hydrogen bonding acceptors and better hydrogen bonding donors, consistent with their dipolar and lipophilic properties.

Thus, on an *ab initio* thermodynamic basis, one would predict that ethyl pyruvate, and its putative enol tautomers, are more likely to partition between a polar aqueous phase and a lipid phase, while retaining conformational stability of the same order as the pyruvate sodium salts. Further, it should be noted that the coordination complex of the pyruvate enolate ester with a divalent cation, such as calcium, shown in Fig. 2 as

structure 6, affords the most pronounced change in properties over pyruvate itself, substantiating the utility of these cation-enolate-ester complexes as promoters of heretofore unexploited reactivities of the pyruvate carbon skeleton conformation.

EXAMPLE 2

Reactivity modeling of pyruvate esters

Searches of the Chemical Abstracts and the ISIS databases (MDL Information Systems, Inc.) were conducted to uncover actual examples of the reactivity of pyruvates and their enolates. While numerous precedents for the reactions of pyruvate salts have been recorded, far fewer examples of the molecular interactions between pyruvate esters and hypervalent oxygen are reported in the organic and biochemical literature. The principal reactions of pyruvates at physiological pH values are hydrate formation (Fig. 2, structure 2) and dimerization to para-pyruvate (Fig. 2, structure 7).

As reported by Margolis et al. (1986), sodium pyruvate at concentrations of 1 Mol/liter or less forms varying amounts of the hydrate and the linear dimer, 4-hydroxy-4-methyl-2-ketoglutaric acid. The hydrate can reach 6-10% and the dimer 20-25% on standing for 48 hrs. This reactivity pattern is an important consideration in the evaluation of sodium pyruvate-containing infusates and perfusates, since the hydrate is unreactive towards hypervalent oxygen and the dimer is an inhibitor of 2-ketoglutarate dehydrogenase, a mitochondrial respiratory enzyme, as well as an inhibitor of glutamate transaminases and lactic acid dehydrogenase. By contrast, neither hydrate formation nor dimerization of

pyruvate esters have been reported in the chemical literature.

While the enol forms of pyruvate are thermodynamically stable in principle, their occurrence in aqueous media is unfavored and half-lives of enolates are measurable only in the 3-5 sec range (Kuo et al. (1979)). As the polarity of the solvent decreases, exemplified by the solvation environment provided by dimethylsulfoxide or dimethylformamide, the half life of the enol increases by at least two orders of magnitude (Chiang et al. (1993); Peliska et al. (1991); Sawyer et al. (1983).

As to reactivity toward hypervalent oxygen, both pyruvate salts and pyruvate esters react to form an initial hydroperoxide intermediate at the carbonyl site, which rearranges by disproportionation to afford acetic acid and carbon dioxide or ethoxycarbonic acid, which undergoes subsequent aqueous solvolysis into carbon dioxide and ethanol (Constantopoulos et al (1984); Sawyer et al. (1983); Starostin et al. (1980)).

However, enolpyruvates can also react by an alternate mechanism that involves addition to the exo-methylene group, as in the case of enolpyruvate C-bromination at the 3-carbon (Sekine et al. (1980)), the chelation controlled addition to allylic compounds (Muderawan et al. (1998)), and the biological addition of carbon dioxide to form oxaloacetate via phosphoenolpyruvate carboxylase (Ausenhus et al. (1992)). Enols of biological ketones in general, as exemplified by D-ring acetyl steroids, react with activated oxygen via the cytochrome P-450 oxidase system to afford hydroxyketones via a transient exomethylene epoxide intermediate (Yamazaki et al. (1997)).

When evaluated on the grounds of thermodynamic likelihood and chemical precedent, pyruvate salts can be predicted, via the REACCS software database correlation system, to react with hypervalent oxygen to afford only decarboxylation to acetate and carbon dioxide. Pyruvate esters, on the other hand, can be expected to afford not only the paired decarboxylation products, acetate and alcohol, but also hydroxylated adducts at the 3-carbon, most probably a 3-hydroxypyruvate. These latter species can again react with hypervalent oxygen to yield glycolic acid and carbon dioxide (Perera et al. (1997)), thereby consuming two equivalents of oxidant.

EXAMPLE 3

Stability and reactivity of pyruvate esters in solution

Based on the foregoing modeling exercises, the following hypothesis driven experiments provide verification in chemical and biological systems and further differentiate the method of this invention from prior art.

Ethyl pyruvate affords a more stable aqueous solution than sodium pyruvate in the presence of calcium salts (Ringer's solution), and this observation can be extended to the study of other pyruvate analogs, as shown in Fig. 1, by dissolving them in Ringer's solution containing at least 0.2 equivalent of calcium per molar equivalent of pyruvate analog titrated with sodium hydroxide, or other suitable inorganic alkali, to physiological pH values. Specifically, the preferred embodiment of this "pyruvated" Ringer's solution for use in NMR, stability, and subsequent biological studies is shown in Table 2. It is to be understood that the pyruvate analog in the instant example

may be substituted with any of the analogs shown in Fig. 1 at any concentration sufficient to afford a homogenous solution or substituted by control substances for comparative purposes, such as pyruvic acid, lactic acid (as would be the case in "lactated" Ringer's solution and other reference or inactive ketoacid analogs. The calcium cation may also be substituted, e.g., with magnesium or any other biologically safe cation capable of substituting for calcium and stabilizing the formation of transient coordination complexes with pyruvate ester enolates in aqueous solution.

TABLE 2

Constituents of Pyruvated Ringer's Solution

Component	*Composition*	*Range*
Isotonic saline	75cc	-- (fixed)
KCl	11.25	-- (fixed)
$CaCl_2$	7.5 mg	5-20 mg
Ethyl pyruvate	0.781 ml	0.5-1.5 ml
NaOH	To pH 7.5	7.35-7.55 (pH)

Following the procedural recommendations for analysis of Margolis et al. (1986) with respect to scanning times and frequencies on a 400 MHz spectrometer operating in pulse-Fourier transform mode, both proton and carbon shifts in the characteristic resonances for each carbon and proton cluster at the enolizable carbon were monitored as a function of time and demonstrated that a greater proportion of pyruvate esters showed a propensity to enolize in Ringer's solutions, especially those containing calcium or magnesium, while pyruvate acid anions showed preponderant hydration and dimerization under similar conditions. The ultraviolet absorptions of these solutions were also measured

periodically over the 230-260 nm range and 300-340 nm span, where changes in enol formation become evident, and provided confirmatory evidence about the distinctly different solvation properties of pyruvate ester analogs in comparison to pyruvate salts applied in the various methods of prior art.

The experimental sequence in which to establish the greater utility of the pyruvate derivatives in this invention follows along the same lines as the comparative spectral experiments just described. The same solutions of test substances used to demonstrate enolization and related phenomena were also used in the comparison of basal values for each candidate pyruvate to the effects of oxidants on the disappearance of characteristic pyruvate resonances and the appearance of acetate or other degradants of the initial test preparation as a function of exposure to these oxidants.

For example, 1 mMolar solutions of pyruvic acid and ethyl pyruvate showed average absorption values, corrected for blanks, of 0.15 and 0.2 respectively at 230-260 nm in the absense of calcium at pH 7.2; addition of calcium had no effect on pyruvate, which showed only a marginal increase in absorption to 0.16, while ethyl pyruvate rose twofold to 0.41 in 3 replicate experiments with a coefficient of variation of less than 15%. When 28 mM solutions were examined in a similar manner at 300-340 nm, the absorbance of pyruvate remained unchanged before and after calcium addition at a value of 0.03, while the ethyl pyruvate solutions become noticeably straw colored to the naked eye, rising in absorbance from 0.07 to 0.85. The yellow coloration and increases in spectrophotometric absorption in

the ultraviolet region confirms the formation of a 1,3-conjugated ketone system, as would result from the enolization of ethylpyruvate under conditions which appear not to enolize pyruvic acid.

Thus, applications of hypervalent oxygen mimics, whose redox potential is known to be a model for ROS, such as hydrogen peroxide, Fenton's reagent, and meta-chloroperbenzoic acid, were dispensed into the test solutions at concentrations ranging from 1 to 50 mMolar and their degradative effects noted. It was shown that pyruvate esters consume a greater proportion of oxidant per molar equivalent than their congeneric free acid analogs.

EXAMPLE 4

Stability and reactivity of pyruvate esters in tissue culture

Pyruvate esters, and in particular ethyl pyruvate, in the presence of calcium ion are sufficiently lipophilic to be taken up by cells at a faster rate than equimolar amounts of pyruvate in the cell preparation perfusate. Moreover, the compounds of this invention serve as prodrugs for intracellular pyruvate delivery and are, therefore, utilized as antioxidants in part by direct decarboxylation of the pyruvate moiety that is delivered intracellularly and made bioavailable after non-specific ester solvolysis by ubiquitous cytosolic carboxylesterases. Prior to hydrolysis intracellularly, these pyruvate esters also react beneficially via enol-mediated, transient epoxidation mediated by hypervalent oxygen, and related toxic oxidants, to form 3-hydropyruvates.

The resulting hydroxypyruvate esters, especially in the case of ethyl pyruvate and its analogs which are depicted in Fig. 1, are then taken up as a metabolic fuel by anapleurotic incorporation, after solvolysis, or subjected to further decarboxylative oxidation by additional equivalents of reactive oxygen species to form the corresponding hydroxyacetates (glyoxylic acids). Thus, it is to be understood that pyruvate esters can quench twice as many reactive oxygen species than the non-enolyzing forms of the corresponding unesterified ketoacid anion; that is, first by the formation of 3-hydroxypyruvates and then by the latter's decarboxylative degradation into a smaller metabolite, which like acetate can be readily incorporated into intermediary metabolism. These outcomes in which the compounds of this invention prove more effective antioxidants, as well as metabolic fuels, after exposure to ROS are demonstrable by combinations of NMR and spectral (UV) analytical procedures that follow, for example, the fate of stable isotope labeled pyruvate[3-^{13}C] species under various experimental conditions.

Accordingly, cell and tissue cultures present a effective means for comparing the relative rates of uptake and subsequent disposition of pyruvate analogs dispensed into the culture or perfusion medium and then monitored for incorporation into cells by means of a stable isotopic tracer that is amenable to proton and carbon magnetic resonance analysis in real time or by mean of mass spectral analysis of suitable extracts of the test biomass after a suitable period of incubation or perfusion.

In particular, since bowel ischemia is one of the more damaging conditions for which pyruvates are known to provide

rescue and resuscitation, the use of enterocyte cell cultures provides a appropriate test model. This model consists of exposing enterocytes after a basal period under various conditions of anoxia and then hyperoxia to a perfusate containing Ringer's solution supplemented with calcium as control and then various tests compositions of pyruvates, including sodium pyruvate, all labeled at the 3-methyl position with ^{13}C. For the carbon MR experiments, cells are seeded on the surface of polystyrene microcarrier beads in bacteriological Petri dishes and grown for 3 days to confluency before harvesting and spectroscopic analysis, following the method of Artemov et al. (1998) and modeling rubrics of Yu et al. (1997) and of Vogt et al. (1997). The test perfusates during the study period are also monitored for purposes of background subtraction from the acquisition of carbon resonances characteristic of the Krebs cycle.

Thus, the rate of carbon flux of exogenously added pyruvate can be followed throughout the process of conversion into citrate and ketoglutarate/glutamic acid. The 3-carbon of pyruvate and the 2-carbon of acetate, derived from pyruvate, are expected to provide differential enrichments at the 2 versus the 4 position of citrate and ketoglutarate. Direct incorporation of the pyruvate carbon skeleton into citrate and ketoglutarate should be expressed as a faster increase in label at the 2 position versus the 4 position, since the latter is more likely to diluted by the larger acetate-acetyl-CoA pool.

If hydroxypyruvate is formed in the reaction, not only can the methyl group resonance be detected directly, but the subsequent utilization of hydroxypyruvate via decarboxylation into glyoxylate and homologation to malate

can also be traced by the same scheme of differential labeling analysis. Experiments of this nature confirm that pyruvate esters act differently as a carbon source from pyruvate salts. Furthermore, such experiments confirm that lactate, acetoacetate and related esters, when substituted for pyruvate esters, do not show enolization and are not incorporated into cells and/or processed via oxidative metabolism in a manner similar to, and to the extent of, the pyruvate esters used in the method of this invention.

EXAMPLE 5

Application of the invention in ischemia rescue

The utility of ethyl pyruvate in a Ringer's solution infusate as a resuscitation fluid in ischemia/reperfusion mucosal injury and barrier dysfunction is demonstrated in this illustrative experiment using a rat model of superior mesenteric artery occlusion. The model system and calculation parameters are illustrated in Fig. 3.

After induction of general anesthesia using intraperitoneal ketamine and pentobarbital, male Sprague-Dawley rats (250-350 g) were subjected to 60 minutes of superior mesenteric artery occlusion followed by 60 minutes of reperfusion. Heart rate and mean arterial blood pressure were measured via a right carotid arterial catheter. The left internal jugular vein was cannulated for intravenous infusions.

Controls (n=6) received lactated Ringer's solution (lactate, 28nM, 111.5 ml/kg/hr infusion, 1.5 ml/kg bolus prior to ischemia, and a 3.0 ml/kg bolus prior to reperfusion). Experimental groups (n=6 each) received similar volumes (3 ml) of either pyruvate, Na salt (28 mM)

or pyruvate ethyl ester (28 mM), prepared in accordance with the method of this invention as shown in Table 2 and at a dosage rate equivalent to 10 mg/kg/hr. Small intestinal mucosal-to-serosal permeability (C_{MS}, nl/min/cm^2) of FITC-dextran (mw = 4 kDA) was evaluated using an everted gut sac technique as previously described by Wattanasirichaigoon (1999). Permeability was measured at baseline, after 30 and 60 minutes of ischemia (I30 and I60) respectively, and after 30 and 60 minutes of reperfusion (R30 and R60, respectively). Histologic samples at baseline, I60 and R60 were evaluated for villous height (VH, μ) and mucosal thickness (MT, μ). Mucosal injury grade was determined according to the method described by Chiu et al. (1970), scored as in Table 3, as follows:

TABLE 3

Mucosal Injury Grade

Grade 0	Normal Mucosa
Grade 1	Subepithelial space formation
Grade 2	Moderate epithelial lifting confined to the tip of the villi
Grade 3	Extensive epithelial lifting, a few tips are denuded
Grade 4	Denuded villi, dilated exposed capillaries, increased cellularity in the lamina propria
Grade 5	Hemorrhagic ulceration

Data were summarized as means ± standard error of the mean. Significances of differences were determined using Student's t-test. Differences were considered significant for $p<0.05$.

The results of these experiments on the utility of the method of invention revealed that both pyruvate

compositions, as free acid as well as ethyl ester, significantly decreased mucosal permeability during reperfusion, as shown in Fig. 4. The ester showed a significant trend towards effecting earlier and greater cytoprotection as judged by the extent of permeability increase, which is a sign of irreversible tissue damage and in terms of the significant diminution in mucosal injury score, shown graphically in Fig. 5. Pyruvate ethyl ester, moreover, significantly maintained villous height and mucosal thickness during both ischemia and reperfusion ($p<0.01$) as shown in Table 4:

TABLE 4

Histological Findings on Beneficial Effects of "Pyruvated" Ringer's Solution

	Lactate		**Pyruvate**		**Pyruvate Ester**	
	VH	MT	VH	MT	VH	MT
Baseline	470±30	553±34	461±25	524±28	486±12	583±8
I60	244±20	298±32	290±30	372±36	381±24§	466±25§
R60	130±25	141±22	201±44	266±50	296±26§	352±34§

Note: Lactate vs Pyruvate and Lactate vs Pyruvate Ester, $p<0.05$ and §$p<0.01$

Taken as a whole, these findings confirm the utility of pyruvate esters in the method of this invention in compositions for the treatment of ischemia and related conditions caused by hypoxia and then reperfusion, with its attendant reactive oxygen damage. The model system described above, a rat model of superior mesenteric artery occlusion, is a standard model system familiar to those of

ordinary skill who wish to provide therapeutic treatment of the kind described, and the results reported above are easily extrapolatable for human use.

Thus, it is apparent that there has been provided, in accordance with the invention, novel 2-ketoalkanoic acid ester compounds and compositions and methods of treating the deleterious effects of hypervalent oxidants resulting from hypoxic damage, followed by reperfusion, that fully satisfies the objects, aims and advantages set forth above.

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While the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications, and variations will be apparent to those skilled in the art in light of the foregoing description. Accordingly, it is intended that the invention shall be directed to all such alternatives, modifications and variations as fall within the spirit and broad scope of the appended claims.

CLAIMS

What is claimed is:

1. A composition comprising an alkyl, aralkyl, alkoxyalkyl or carboxyalkyl ester of 2-ketoalkanoic acid in a pharmaceutically-acceptable carrier, said carrier further comprising a biologically safe component for inducing and stabilizing enolization of the 2-keto functionality of said ester at physiological pH values, wherein, in said carrier, the enol form of said 2-keto functionality of said ester is stabilized at physiological pH values.

2. The composition of claim 1, wherein said component for inducing and stabilizing enolization of the 2-keto functionality of said ester is an inorganic, divalent cation.

3. The composition of claim 2, wherein said divalent cation is calcium or magnesium.

4. The composition of claim 1, wherein said 2-ketoalkanoic acid portion of said ester is 2-ketopropionic acid.

5. The composition of claim 1, wherein said 2-ketoalkanoic acid ester is the ethyl ester.

6. The composition of claim 2, wherein said 2-ketoalkanoic acid ester is ethyl pyruvate, said divalent

cation is calcium and said pharmaceutically-acceptable carrier is Ringer's solution in a pH range of 7-8.

7. A method for administering a therapeutically effective compound to a mammal, said method comprising intraluminally or intravenously administering to said mammal a therapeutically effective amount of a composition comprising an alkyl, aralkyl, alkoxyalkyl or carboxyalkyl ester of 2-ketoalkanoic acid in a pharmaceutically-acceptable carrier, said carrier further comprising a biologically safe component for inducing and stabilizing enolization of the 2-keto functionality of said ester at physiological pH values, wherein said ester is therapeutic as an anti-oxidant and an intracellular fuel for said mammal.

8. The method of claim 7, wherein said pharmaceutically-acceptable carrier is a Ringer's solution of isotonic saline supplemented with potassium ion.

9. The method of claim 7, wherein said 2-ketoalkanoic acid ester is selected from the group consisting of ethyl pyruvate, propyl pyruvate, butyl pyruvate, carboxymethyl pyruvate, acetoxymethyl pyruvate, carbethoxymethyl pyruvate and ethoxymethyl pyruvate.

10. The method of claim 7, wherein said 2-ketoalkanoic acid ester is selected from the group consisting of ethyl 2-keto-butyrate, ethyl 2-ketopentanoate, ethyl 2-keto-3-methyl-butyrate, ethyl 2-keto-4-methyl-pentanoate and ethyl 2-keto-hexanoate.

11. The method of claim 9, wherein said 2-ketoalkanoic acid ester is admixed in a saline solution, said solution containing a cation selected from the group consisting of calcium and magnesium.

12. The method of claim 10, wherein said 2-ketoalkanoic acid ester is admixed in a saline solution, said solution containing a cation selected from the group consisting of calcium and magnesium.

13. The method of claim 7, wherein said composition is administered to treat events of mesenteric ischemia, mesenteric thrombus, mesenteric venous occlusion, aortic aneurism repair, coronary artery bypass, or surgical treatment of arterial occlusion of limbs.

14. A process for preserving organ parts, organs or limbs removed from a living mammal, said process comprising perfusing said organ with a solution containing an effective amount of a composition comprising an alkyl, aralkyl, alkoxyalkyl or carboxyalkyl ester of 2-ketoalkanoic acid in a pharmaceutically-acceptable carrier, said carrier further comprising a biologically safe component for inducing and stabilizing enolization of the 2-keto functionality of said ester at physiological pH values.

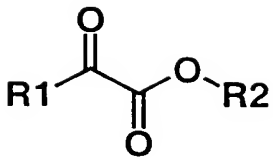
15. The process of claim 14, wherein said 2-ketoalkanoic acid ester is selected from the group consisting of ethyl pyruvate, propyl pyruvate, butyl pyruvate, carboxymethyl

pyruvate, acetoxymethyl pyruvate, carbethoxymethyl pyruvate and ethoxymethyl pyruvate.

16. The process of claim 14, wherein said 2-ketoalkanoic acid ester is selected from the group consisting of ethyl 2-keto-butyrate, ethyl 2-ketopentanoate, ethyl 2-keto-3-methyl-butyrate, ethyl 2-keto-4-methyl-pentanoate and ethyl 2-keto-hexanoate.

17. The process of claim 15, wherein said 2-ketoalkanoic acid ester is admixed in a saline solution, said solution containing a cation selected from the group consisting of calcium and magnesium.

18. The process of claim 16, wherein said 2-ketoalkanoic acid ester is admixed in a saline solution, said solution containing a cation selected from the group consisting of calcium and magnesium.

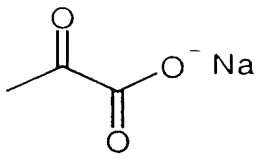


R_1 = Methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tertbutyl, pentyl, 4-methylpentyl, 3-methylpentyl, hexyl, heptyl, octyl, 1-phenylmethyl, 2-phenyl-ethyl;

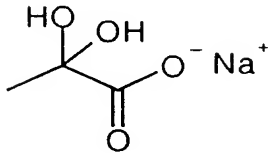
R_2 = Ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tertbutyl, pentyl, 4-methylpentyl, ethoxymethyl, 2-ethoxyethyl, carboxymethyl, ethoxycarbonylmethyl.

FIG. 1

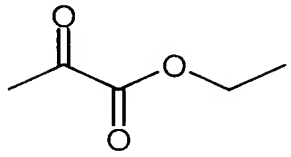
1) Na Pyruvate



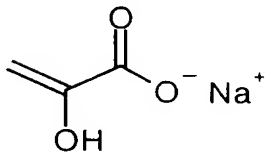
2) Na Pyruvate hydrate



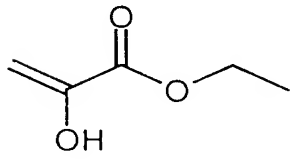
3) Ethyl pyruvate



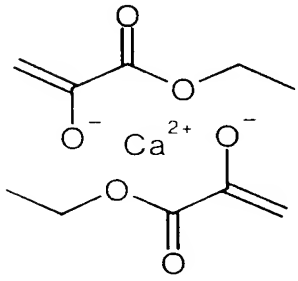
4) Na enolpyruvate



5) Ethyl enolpyruvate



6) Ca enol ethyl ester



7) Parapyruvate

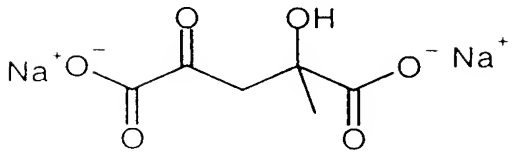


FIG. 2

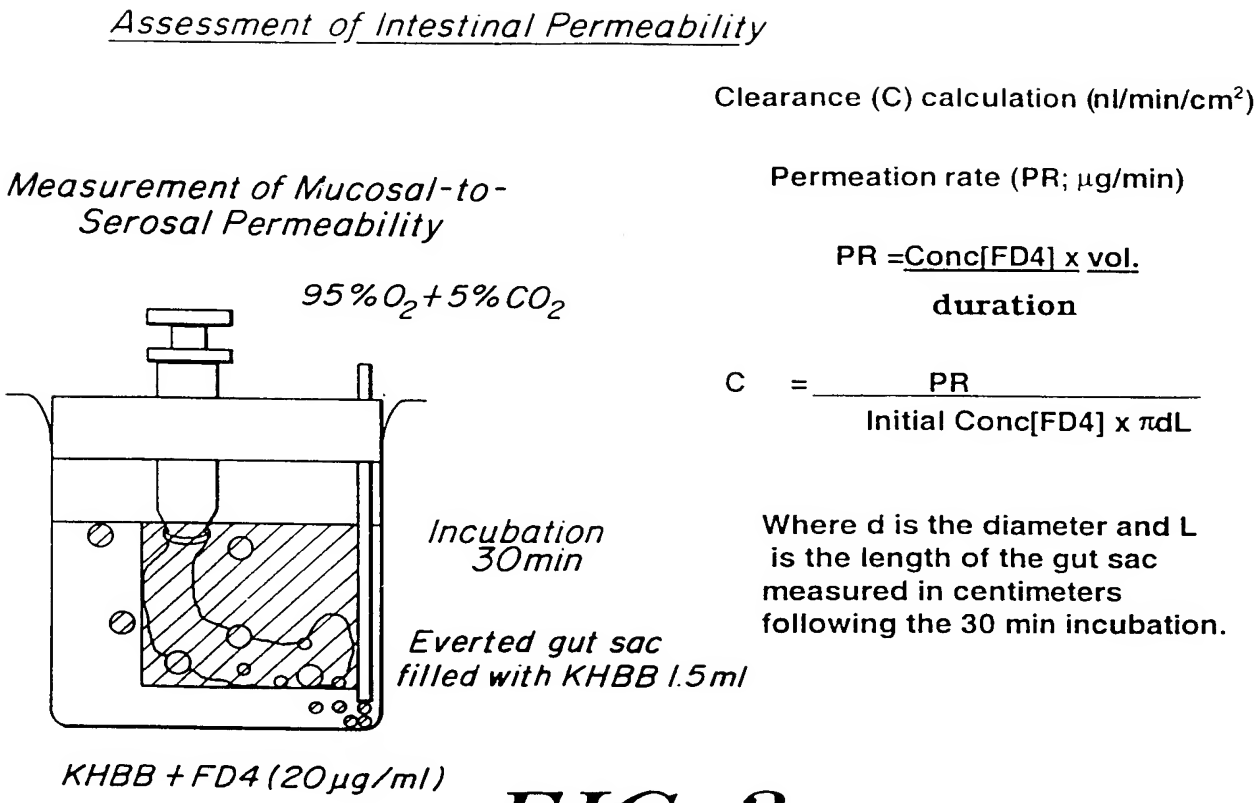

Assessment of Intestinal Permeability
Clearance (C) calculation (nl/min/cm²)
Measurement of Mucosal-to-Serosal Permeability
95%O2+5%CO2
Permeation rate (PR; µg/min)
PR =Conc[FD4] x vol. / duration
C = PR / Initial Conc[FD4] x πdL
Incubation 30min
Where d is the diameter and L is the length of the gut sac measured in centimeters following the 30 min incubation.
Everted gut sac filled with KHBB 1.5ml
KHBB + FD4 (20µg/ml)

FIG. 3

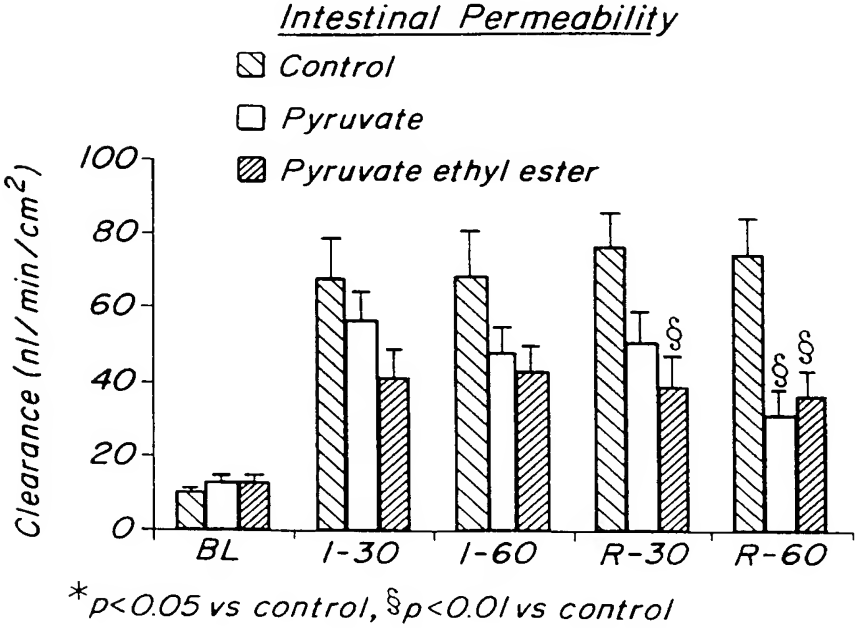

Intestinal Permeability
Control
Pyruvate
Pyruvate ethyl ester
Clearance (nl/min/cm²)
100
80
60
40
20
0
BL
I-30
I-60
R-30
R-60
*p<0.05 vs control, §p<0.01 vs control

FIG. 4

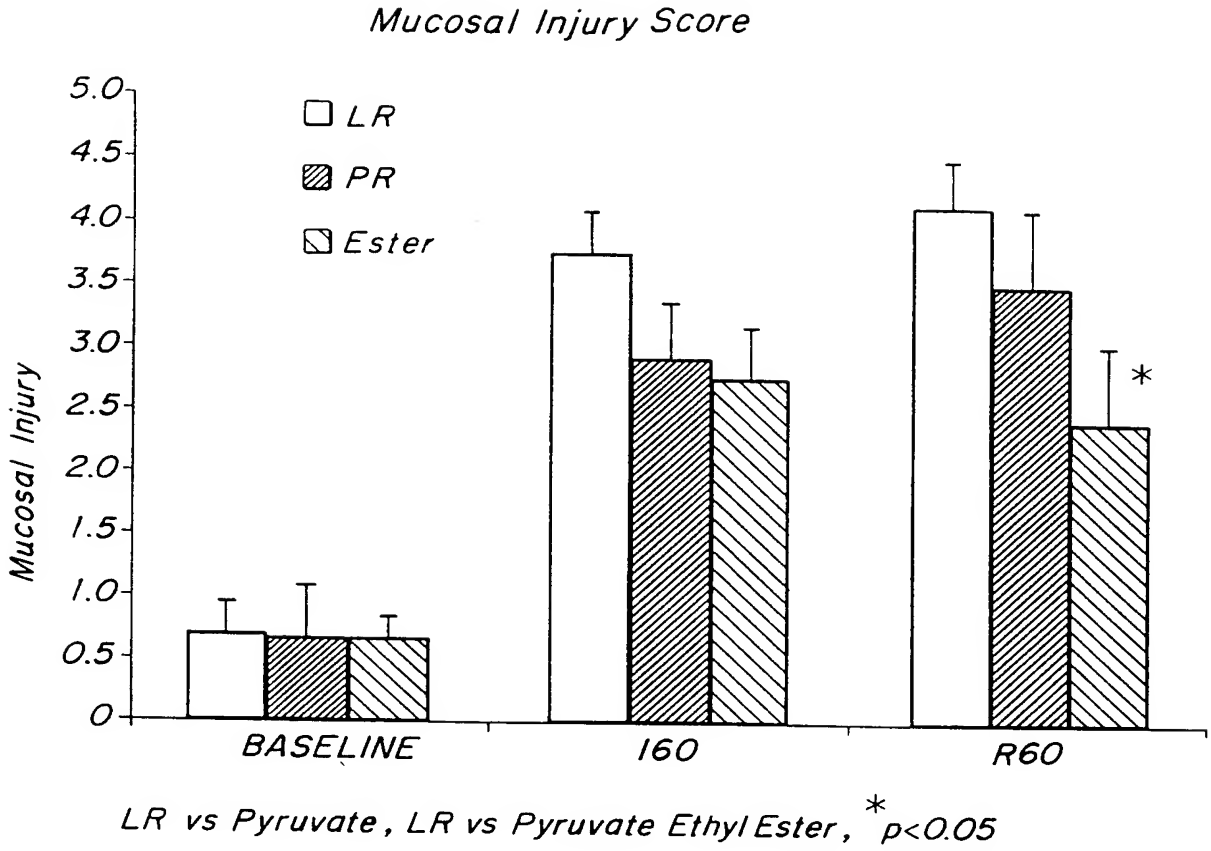

Mucosal Injury Score
Mucosal Injury
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
LR
PR
Ester
*
BASELINE
160
R60
LR vs Pyruvate, LR vs Pyruvate Ethyl Ester, * p<0.05

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/27758

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/235

US CL : 514/533, 921

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/533, 921

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,075,210 (WIKMAN-COFFELT) 24 December 1991 (24.12.1991), see abstract, column 1, lines 6-20, column 7, lines 15-62, column 9, line 28 to column 10, line 13)	1-18
Y	US 5,876,916 A (BRUNENGRABER et al.) 02 March 1999 (02.03.1999), see abstract, column 1, lines 9-67, column 2, lines 41-47, column 2, line 64 to column 3, line 16, column 4, lines 36-41, column 5, lines 18-51,	1-18
Y,P	US 5,968,727 A (BRUNEGRABER et al.) 19 October 1999 (19.10.1999) see abstract, column 1, lines 10-47, column 2, line 41to column 3, line 15, column 4, lines 47-55, column 5, lines 23-57, column 7, lines 34-45,	1-18
Y,P	US 6,086,789 A (BRUNENGRABER et al.) 11 July 2000 (11.07.2000) see column 1, lines 15-33, column 1, lines 31-42, column 2, line 51 to column 3, line 6, column 6, lines 34-67, column 8, lines 50-58	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 December 2000 (18.12.2000)

Date of mailing of the international search report

25 JAN 2001

Name and mailing address of the ISA/US

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Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/27758

Continuation of B. FIELDS SEARCHED Item 3: WEST 2.0, STN files ADISALERTS, ADISINSIGHT, BIOSIS, BIOTECHNO, CANCERLIT, CAPLUS, CEN, DDFB, DDFU, DGENE, DRUGB, DRUGLAUNCH, DRUGMONOG2, DRUGNL, DRUGU, EMBAL, EMBASE, ESBIOBASE, IFIPAT, IPA, JICST-EPLUS, KOSMET, LIFESCI, MEDICONF, MEDLINE, NAPRALERT, NLDB, PHIC, PHIN, SCISEARCH, TOXLINE, TOXLIT, USPATFULL. Search Terms: ajami, sims, fink, transplant?, reperfus?, ischem?, pyruvate ester, calcium, magnesium ringers.